PHOSPHATASES OF THE HOUSE FLY, MUSCA DOMESTICA

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The biological role of the monophosphoesterases in metabolism of proteins, in the possible dephosphorylation of certain hexose phosphates, in bone formation and in active transport across a gradient barrier in living organisms has been thoroughly reviewed by Moog (1). The relatively limited literature concerning the importance of these enzymes in insects more specifically has been reviewed by Fitzgerald (2) and by Rockstein and Herron (3). Since that time, comparative data on the quantitative differences in activity of alkaline and acid glycerophosphatase in six species of insects have appeared by this author and Levine (4) and with Inashima (5).

In an extended investigation of certain aspects of intermediary metabolism in the adult house fly, a preliminary study was undertaken to establish certain features of the chemistry of and optimal conditions for studying these enzymes in this insect. This report represents a summary of such data.

Experimental[1]

Enzyme activity was studied by modifications of methods described earlier (3), in which total homogenates are incubated with substrate in the presence of Mg ions, at 35° C., following which released phosphate is estimated in aliquots of the deproteinized incubation mixture.

Homogenizing Procedure: Adult house flies 24 hours old were inactivated by rapid freezing (6) and stored at −20°C. until needed. Twenty male flies were homogenized with 1 ml. of ice-cold water for three minutes in a mechanically-driven Elvehjem-Potter glass homogenizer (with Teflon pestle) in an ice-bath. The homogenate was decanted and the pestle and cup each washed three times with one-ml. quantities of cold water (deionized) onto a 20 mg. disc of washed glass wool (Pyrex 800) exactly fitting the 25-mm. inner diameter of a Hirsch (Coors 000A) funnel. The funnel was rinsed with 1-ml. volumes of cold water. The filtrate was collected under moderate suction directly into a 10 ml. volumetric flask by means

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[1] All procedures preceding incubation were done in the cold room at 5°C.; deionized water was employed in preparations of reagents or homogenate suspensions.

of a microsuction flask, for 10 minutes, and diluted to mark with cold water. Preceded each time by mixing by inversion three times, 0.5 ml. aliquots of diluted homogenate were delivered into individual shell vials, which later served as incubation tubes. These were either used immediately or stored frozen at −20°C.

Enzyme Procedure: Into each vial containing 0.5 ml. homogenate, was delivered 0.5 ml. of ice-cold 0.5M Na_2 beta-glycerophosphate (Eastman Kodak) dissolved in 0.424 per cent sodium diethyl barbiturate (barbital) (Merck) buffer, 0.25 ml. of 0.8M $MgCl_2$, plus a drop of chloroform.[2] The stoppered vials were inverted three times for complete mixing, placed in a constant temperature water bath and incubated for 25 minutes, in addition to an initial 5-minute warm-up period. Controls (one for each experimental) consisted of incubation mixture identical with experimental mixtures, except that they were inactivated exactly at the end of the 5-minute warm-up period.[3] Inactivation (and deproteinization) was effected at the termination of the incubation period by the rapid addition to each ml. of incubation mixture of 0.2 ml. of 30 per cent trichloroacetic acid; this was followed by complete mixing, a 5-minute wait, and filtration for 7 minutes through a (4.25 cm.) disc of (No. 42 Whatman) filter paper folded into a small (one-inch) glass funnel. Phosphate content of 0.2 ml. samples of this deproteinized filtrate was estimated by the author's adaptation (7) (to microgram estimation) of Sumner's ferrous sulfate-acid molybdate method for determining inorganic phosphate. Absorbance was read at 720 mμ in the Beckman (DU) spectrophotometer; final activity was calculated from the net absorbance (experimental minus control value) in relation to that of the standard phosphate sample.

Results[4]

[2] For each set of determinations concerned with variation in a particular parameter, conditions were exactly as stated, except for the particular variable under consideration. For details see legend of each figure (below).

[3] Preliminary determinations established that there is no significant difference between controls, identical with experimentals except for prior inactivation by heating, and such "zero-time" controls.

[4] Activity is expressed in micrograms P released 25 min 0.2 ml. deproteinized incubation mixture, at 35°C. (except where time or temperature has been varied). Unless otherwise stated each plotted point in Figs. 1–5 represents the median value from five to eight sets of determinations.

Enzyme Concentration: Figure 1[5] shows a direct proportionality between activity and enzyme concentration, expressed as ml. of homogenate. All samples of this homogenate less than the usual 0.5 ml. were made to 0.5 ml. by addition of appropriate volumes of deionized water. On the basis of this study, for all future experiments 0.5 ml. volumes of diluted brei from homogenization of 20 flies were employed, each of the 0.5 ml. aliquots representing the

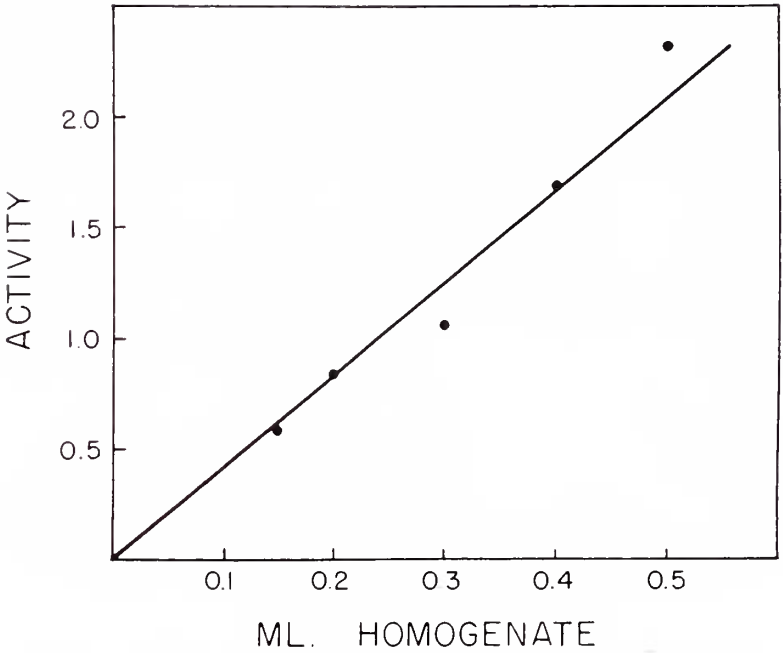

Fig. 1. Alkaline phosphatase activity and enzyme concentration; variable homogenate (30 male flies, 24 hrs. old, per 10 ml.), 0.5 ml. 0.5M substrate, 0.25 ml. 0.8M $MgCl_2$, pH 8.4; incubation time 25 min. at 35°C.

filtered and diluted homogenate from one fly (of 10 mg. average fresh weight) of known sex and age.

Substrate Concentration: Figure 2 shows the effects of increasing concentration of substrate[6] upon the enzyme activity, as the

[5] Straight line of figures 1 and 5 were drawn by the method of least squares.

[6] Serial dilutions were made with 0.424 per cent Na barbital buffer solution.

latter is maintained constant. Activity is seen to rise rapidly with increasing substrate concentration especially up to about 0.5M after which a decline in rate of increase in velocity is observed.

Varying pH: Figure 3 shows the effect of varying pH by the

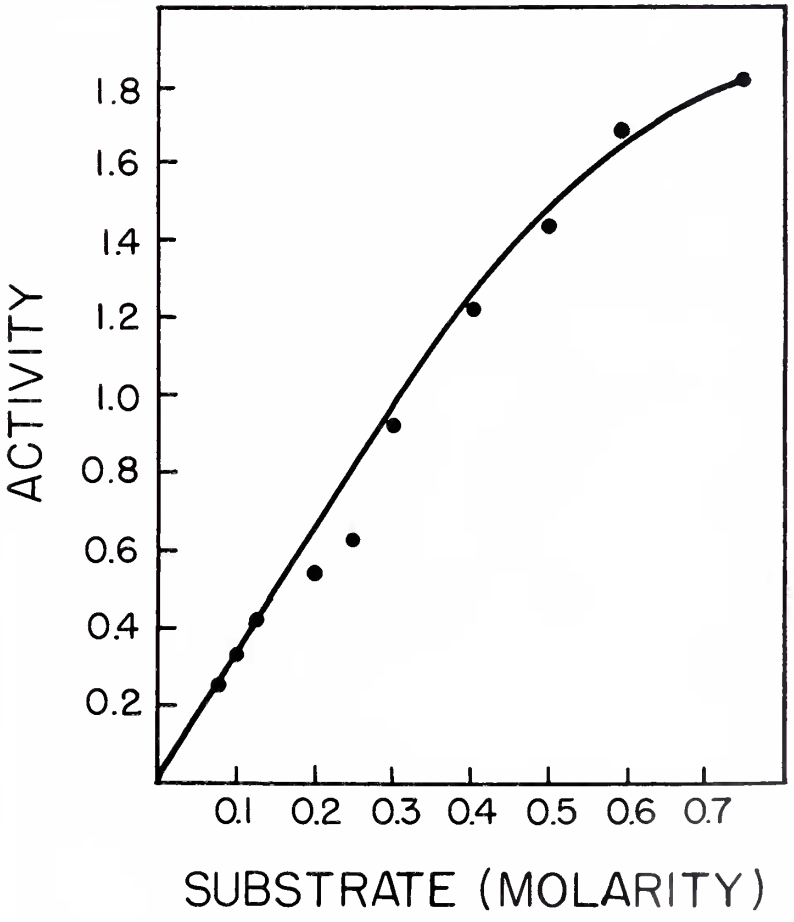

Fig. 2. Alkaline phosphatase activity and substrate concentration; conditions exactly as in Fig. 1, except substrate varied from 0.075 M- 0.75M- each dissolved in 0.424 per cent barbital buffer solution, homogenate 0.5 ml. (20 male flies/10 ml., 24 hrs. old).

addition of varying amounts of acetic acid or NaOH to an incubation mixture as described for the figure. A typical pH activity

curve is seen, except that at pH above 9.5 no further data could be obtained because of the formation of a white precipitate at higher pH values; optimum pH in the acid range is 5.7 with a secondary alkaline maximum somewhere above 9.0. (Table 1 shows the relation of pH of incubating mixture to acid or base added.)

Kinetics: Figure 4 shows the change in activity, expressed as

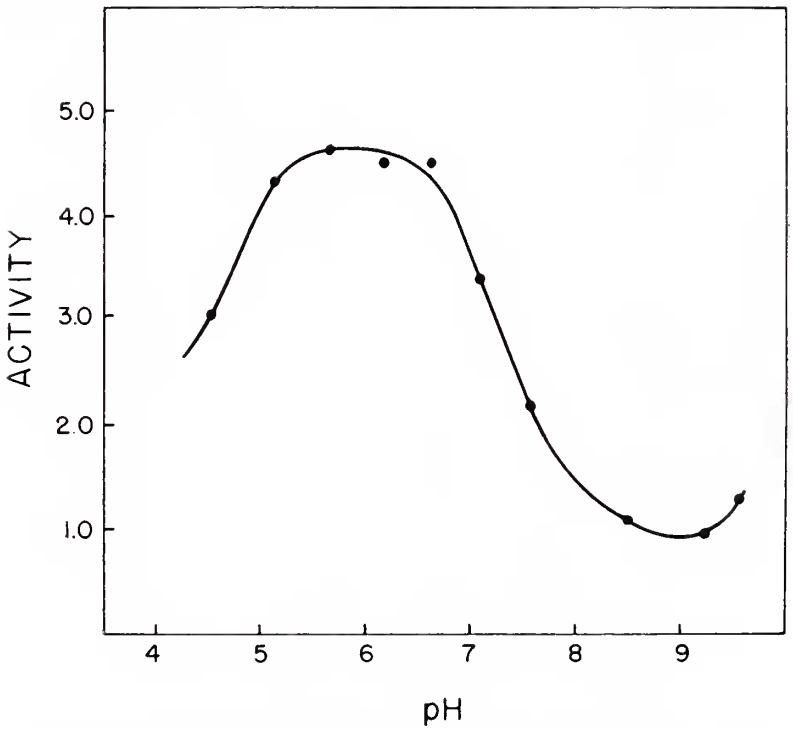

Fig. 3. Phosphatase activity and pH; 2 ml. homogenate (20 males/10 ml., 24 hrs. old), 0.8 ml. 1M substrate in 0.848 per cent Na barbital buffer solution, variable AcOH or NaOH (as shown in Table 1), 1 ml. 0.8M Mg Cl_2, pH variable from 4.5–9.5, incubation time 25 min. at 35°C.

total phosphorus released for each stated interval of time. For the first 40 minutes of time, under the particular conditions of enzyme study, a zero order reaction (*i.e.,* activity per unit time is constant) is seen to prevail. This, of course, indicates that the substrate is maintained in excess, with the (theoretical) substrate-enzyme com-

plex determining the speed of reaction. In this connection, it should be emphasized that preliminary studies of the kinetics of the alkaline enzyme were made for each of the previous studies, except for the pH study, to insure working under such conditions that substrate remained in excess and a zero order reaction was thus maintained for the limits of each experiment.

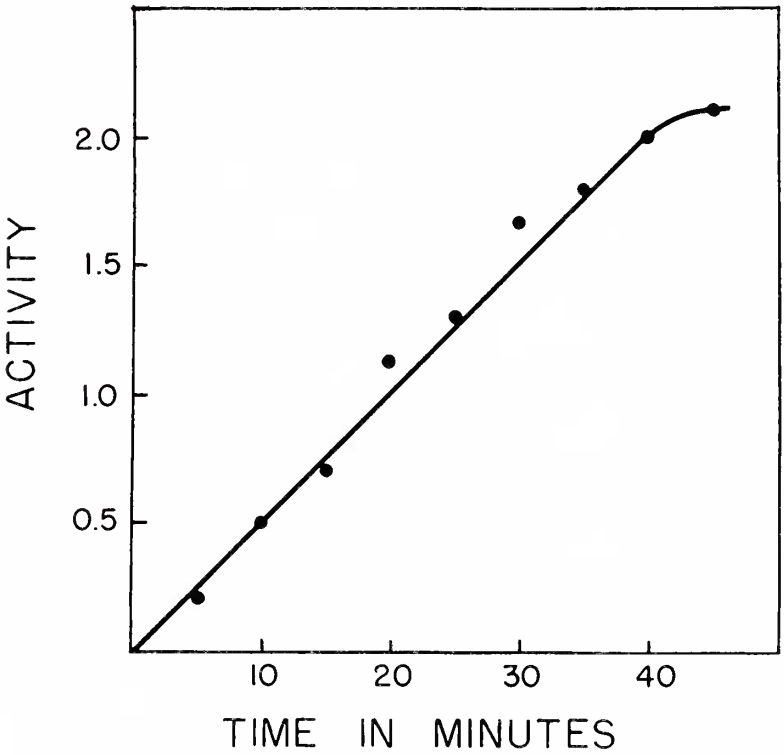

Fig. 4. Kinetics of alkaline phosphatase; conditions exactly as in Fig. 1, except 0.5 ml. of similar homogenate (20 males 10 ml., 24 hrs. old) and time varied from 5 to 45 min.

Temperature Effects: Figure 5 shows the effects of varying temperature upon enzyme activity, under time, enzyme and substrate conditions established to be optimal earlier. A representative curve is seen, with activity falling off above 40°C., the optimal temperature, which fall-off suggests inactivation of the enzyme itself. Below 20°C. activity was too low to be estimated, even by this sensitive colorimetric method.

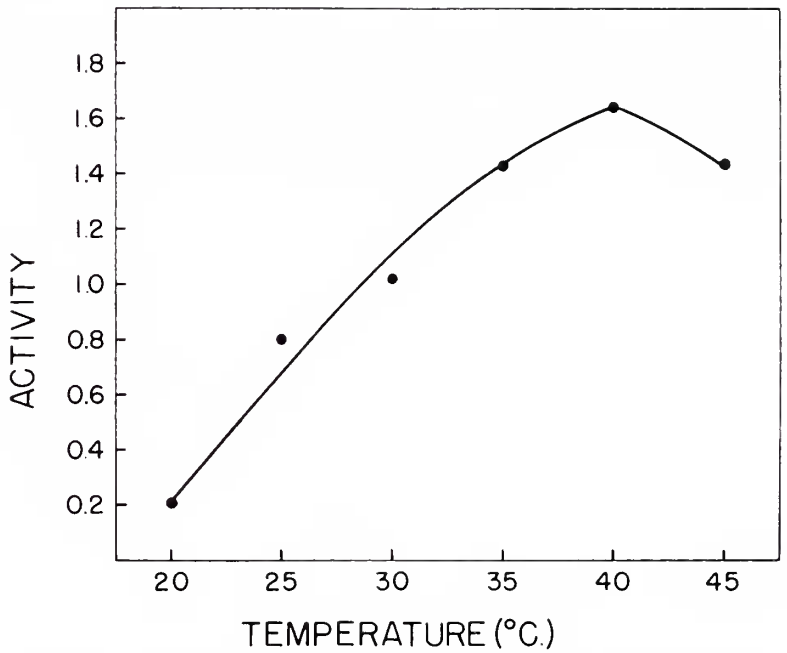

Fig. 5. Temperature and alkaline phosphatase; conditions as above, for 25 min. incubation periods at varying temperatures, as indicated.

Table 1. The pH of incubating mixture* with addition of acid or base

Acid or Base Added	pH
1.2 ml. 1N AcOH	4.5
0.7 ml. 1N AcOH	5.1
0.4 ml. 1N AcOH	5.7
0.2 ml. 1N AcOH	6.2
0.4 ml. 0.25N AcOH	6.6
0.2 ml. 0.25N AcOH	7.1
0.1 ml. 0.25N AcOH	7.6
1.2 ml. H_2O (deionized)	8.4
0.2 ml. 0.1N NaOH	9.2
0.8 ml. 0.1N NaOH	9.5

* 1M substrate in 0.848% barbital solution; where less than 1.2 ml. of acid or base was added, this total volume was obtained by addition of sufficient cold water.

Activating Ions: Table 2 shows the effects of varying concentrations of Mg ions and of employing other divalent cations in incubation mixtures for alkaline phosphatase estimation. pH values did not vary significantly for the various mixtures containing different $MgCl_2$ concentrations; the pH values for mixtures containing other

Table 2.† The effect of Mg^{++} and other divalent ions on alkaline enzyme activity

Ions		Net Absorbance
Series 1—$MgCl_2$	0.01M	0.019
	0.08M	0.023
	0.40M	0.025
	0.80M	0.029
	2.00M	0.027
Series 2—$MgCl_2$	0.20M	0.021
	0.40M	0.034
	0.80M	0.030
	1.00M	0.030
	2.00M	0.028
Series 3—$MgCl_2$	0.40M	0.040
	0.60M	0.040
	0.80M	0.041
	1.00M	0.042
	1.50M	0.035
	2.00M	0.025
Series 4—0.80M	$MnCl_2$ (7.8)	0.084
	$CoCl_2$ (7.4)	0.085
	$FeCl_2$ (—)	0.126
	$MgCl_2$ (9.0)	0.125
	$CaCl_2$ (8.5)	0.018

† Incubation mixture—0.5 ml. of homogenate (20 flies 10 ml.), 0.5 ml substrate (0.5M in 0.424 per cent barbital for all but series 4, where 1M in 0.848 per cent barbital buffer was used), 0.25 ml. of solution of divalent ions; incubated at 35°C. for 25 min.

cations are shown in parentheses. Original concentrations of ions are given for the 0.5 ml. volumes added. Each absorbance value is the median of three different determinations; for all incubation mixtures of each series of determinations, aliquots of the same homogenate were employed.

Discussion

Inasmuch as the major objective of this series of studies was a quantitative characterization and classification of a monophosphoesterase system concerned with splitting Na beta-glycerophosphate, the results obtained place the alkaline enzyme in class A1 of Folley and Kay (8, 9) or type I of Roche (10) on the basis of pH optimum and Mg ion activation, as in the case of the honey bee (3). However, purely in terms of pH optima, the alkaline enzyme would fall into category 1 and the acid enzyme into category 3 of the original Bamann-Meisenheimer classification of these phosphatases (11). As Roche states, crude tissue extracts, containing mixtures of isodynamic enzymes, are difficult to classify correctly in view of the uncertain comparative purity of different preparations. Nevertheless, it is significant that the alkaline enzyme of house fly extracts has a similar pH optimum and is activated strongly by Mg, Co and Mn but not by Ca ions like Roche's type I phosphomonesterase.

The optimal temperature of 40°C. found here corresponds closely to that reported previously for alkaline phosphatase in a number of other invertebrates (12) and in mammals by Bodansky (13).

Kinetics data agree well with those previously reported by Fitzgerald (2) and by the present author (3) for alkaline phosphatase as well as for hydrolytic enzymes in general by Van Slyke (14). Under the conditions described above, for further quantitative study of this enzyme system in the adult house fly it is suggested that a maximum of 30 minutes incubation be employed to insure the maintenance of a zero order reaction.

The possible role of this enzyme system in the normal biochemistry of the insect species in question has been suggested at length earlier by the present author (3, 15) in relation to previously reported correlative changes in glycogen content and motor ability with physiological fatigue in flying insects. Recent findings by this author (to be published) show a strong parallelism in quantitative distribution of this phosphatase system and of adenosine triphosphatase in the adult house fly, in relation to development, maturation and aging.

Based on the details of optima for the various parameters considered in this report, the following recommended conditions are currently being employed in a study concerned with certain aspects of phosphorus metabolism in the adult house fly:

Alkaline Enzyme	Acid Enzyme
0.5 ml. homogenate (as described)	0.5 ml. homogenate (as described)
0.25 ml. 0.8M $MgCl_2$	0.25 ml. 0.8M $MgCl_2$
0.5 ml. 0.5M substrate in 0.424% Na barbital	0.2 ml. 1M substrate in 0.828 % Na barbital
	0.3 ml. 0.33N AcOH

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Summary

1. Details of preparation and study of an enzyme system hydrolyzing Na beta-glycerophosphate are presented, for total tissue extracts of the adult house fly.

2. A pronounced maximum activity was seen at pH 5.7 and a second maximum suggested somewhat above pH 9.0. Acid activtiy was considerably higher than alkaline.

3. Under the stated conditions of study a zero order reaction obtained up to 40 minutes of incubation.

4. The optimal temperature for conditions stated was 40°C.

5. Mg, Mn, Co and Fe ions strongly activated the alkaline enzyme; Ca ions were relatively ineffective as activators.

6. Based on these data, recommended conditions of study of alkaline and acid enzyme are presented.

Literature Cited

1. **Moog, F.** 1946. Biol. Rev. 21: 41.
2. **Fitzgerald, L. R.** 1949. J. Exptl. Zool. 110: 461.
3. **Rockstein, M. and P. W. Herron.** 1951. J. Cell. Comp. Physiol. 38: 451.
4. ——— **and L. Levine.** 1951. Annals Entomol. Soc. Amer. 44: 469.
5. ——— **and M. D. Inashima.** 1953. Bull. Bklyn. Entomol. Soc. 48: 20.
6. ———. 1953. Annals Entomol. Soc. Amer. 46: 544
7. ——— **and P. W. Herron.** 1951. Analytical Chem 23: 1500.
8. **Folley, S. J. and H. Kay.** 1936. Ergebnisse d. Enzymforsch. 5: 159.
9. ———. 1937. *Tabulae Biologicae* 12: 268.
10. **Roche, J.** 1950. *The Enzymes* (Eds., Sumner, J. B. and K. Myrbäck), Vol. I, Pt. 1, Ch. 11.
11. **Bamann, E. and N. Meisenheimer.** 1941. Bamann-Myrbäck's *Methoden der Fermentforschung.* 2: 1623.
12. **Lipman, H. J.** 1940. Bull. Univ. Pittsburgh 15: 175.
13. **Bodansky, O.** 1939. J. Biol. Chem. 129: 197.
14. **Van Slyke, D. D.** 1942. Adv. Enzymol. 2: 33.
15. **Rockstein, M.** 1953. Biol. Bull. 105: 54.